Subject: Response to the ICCVAM Request for Information on Alternative Skin Sensitization Methods and Comment on Proposed Activities

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To: NIEHS NICEATM

Please find below information about 2 tests that are curently being evaluated by industry with the purpose of establishing a validation report to be submitted to EURL-ECVAM.

The tests described below fit in the Mode of Action pathway (Adler et al., 2011. Arch. Toxicol. DOI 10.1007/s00204-011-0693-2) and the Adverse Outcome pathway (OECD, 2013) by addressing epidermal inflammation (RHE IL-18 potency test), dendritic cell (DC) activation (GARD test) and DC migration (DC migration test).

1. The reconstructed human epidermis (RHE) IL-18 potency test

This test builds upon 2 assays (www.Sens-it-iv.eu, Roggen and Blaauboer (2013) Toxicology In Vitro, DOI:10.1016/j.tiv.2013.01.009) which in a two-tiered set-up predict the potency class of skin sensitizers (concordance = 92%) (dos Santos et al. (2011) Toxicology In Vitro 25: 347-357; Galbiati and Corsini (2012) Current Protocols in Toxicology 54: 20.8.1-18). One disadvantage of this tiered approach is that it cannot assess chemicals that are not sufficiently soluble or stable in an aqueous environment.

The RHE IL-18 potency test combines both assaysand allows for the asessment of compounds with limited water solubility/stability. A protocol was developed using different 3D-epidermal models including in house VUMC model, epiCS® (previously EST1000TM), MatTek EpiDermTM and SkinEthicTM RHE and also the impact of different vehicles (acetone:olive oil 4:1, 1% DMSO, ethanol, water) was investigated. Following topical exposure for 24 h to 17 contact allergens and 13 non-sensitizers a robust increase in IL-18 release was observed only after exposure to contact allergens. A putative prediction model was proposed from data obtained from two laboratories yielding 95% accuracy. Correlating the in vitro EE sensitizer potency data, which assesses the chemical concentration which results in 50% cytotoxicity (EE-EC50) with human and animal data showed a superior correlation with human DSA05 (µg/cm2) data (spearman r=0.8500; P value (two-tailed) = 0.0061) compared to LLNA data (spearman r=0.5968; P value (two-tailed) = 0.0542). DSA05 = induction dose per skin area that produces a positive response in 5% of the tested population Also a good correlation was observed for release of IL-18 (SI-2) into culture supernatants with human DSA05 data (spearman r=0.8333; P value (two-tailed) = 0.0154) (Gibbs et al., 2013. Toxicol. Applied Pharmacol. DOI:10.1016/j.taap.2013.07.003).

This easily transferable human *in vitro* assay appears to be very promising, but additional testing of a larger chemical set with the different EE models is required to fully evaluate the utility of this assay and to establish a definitive prediction model. At present 7 industry groups and the 2 academic test developers are producing data to further define the quality, strengths and limitations of the test.

2. The Genomic Allergen Rapid Detection (GARD) test

Functional and transcriptional analysis various myeloid cell lines has clearly demonstrated that the MUTZ-3 cells (i) have ability to induce antigen-independent proliferation in CD4(+) CD45RA(+) T cells, whereas KG-1 and THP-1 only induce a marginal response, (ii) display phenotypic and transcriptional profiles of immature DCs upon differentiation with granulocyte-macrophage colony-stimulating factor and interleukin-4, and (iii) express a mature phenotype and gene induction profile similar to that of monocyte-derived DCs upon activation with inflammatory cytokines. This delineation of the cellular and transcriptional activity of MUTZ-3, in response to maturational stimuli, demonstrates the significance of this cell line as a model for functional studies of inflammatory responses (Larsson et al., 2006. Immunol. 117: 156-166; Lundberg et al., PLoS ONE 8: e52875).

Extensive genomic analysis of MUTZ-3 cells exposed to 80 chemicals has identified a subset of 200 genes that are affected specifically by skin sensitizers and not by respiratory sensitizers or irritants. Using a Support Vector Machine for supervised classification, the prediction performance of the assay revealed an area under the ROC curve of 0.98. In addition, categorizing the chemicals according to the LLNA assay, this gene signature could also predict sensitizing potency. The identified markers describe eight dominating functions: (i) small molecule biochemistry, (ii) cell death, (iii) lipid metabolism, (iv) hematological system development, (v) cell cycle, (vi) molecular transport, (vii) cellular growth, proliferation and development, and (viii) carbohydrate metabolism. At this point it is worthwhile mentioning that well-known markers for sensitization such as CD86, CD80, CD54, CXCL8, IL-1β, MIP-1β and p38 MAPK are relevant but to every sensitizer. (Johansson et al., 2011. BMC Genomics 8:12).

The predictive performance of the assay has been estimated in an in-house validation study. Blind samples were provided by third parties, and GARD were used to assess the unknown chemicals as sensitizers or non-sensitizer.I n total, 33 out of 37 chemicals were correctly classified, yielding a prediction accuracy of 90%. The test data set contained chemicals that are prone to be misclassified in both in vivo and in vitro assays, and thus, GARD has demonstrated to be a compelling alternative to current animal-based assessment of sensitizers (Lindstedt et al., 2011. Biomark Med. 5: 6; Johansson et al., 2013. Toxicol. In Vitro 27: 3)

Using the GARD setup, the potency of sensitizers has successfully been correlated to the engagement of differentially regulated pathways in subgroups of sensitizers. Furthermore, separate biomarker signatures that identifies chemical respiratory sensitizers have been identified, making GARD a unique assay able to provide simultaneous assessment of dual end-points.

3. DC migration test

Fibroblasts play a key role both as advisors helping the KCs and LCs to discriminate irritants from sensitizers, which in many cases are irritants themselves, and as guides helping the LCs out of the epidermis into the dermis and further towards lymphatic vessels (Ouwehand et al., 2012. Eur. J. Immunol. 40: 2026-2034). Using a full-thickness tissue-engineered skin model containing fully functional MUTZ-3 derived LCs (MUTZ-LC), Ouwehand and coworkers (2011. Technical Advance 90: 1028-1033) demonstrated that the MUTZ-LCs mature and acquire the ability to migrate toward CXCL12 and CCL19/21 in a comparable manner with primary LCs in full-thickness skin explants.

The acquired knowledge has resulted in a DC-migration assay which is based on CFSE (carboxyfluorescein succinimidyl ester) labelled MUTZ-3 cells. The discriminating feature of the assay is that irritant-induced migration is CCL5 dependent, while sensitizer-induced migration is CXCL12 dependent. The read-out of the test is the ratio between migration towards CXCL12 or to CCL5. In spite of its complexicty, it seems to be relatively well transferable as demonstrated by Rees and coworkers (2011. Toxicol In Vitro 25: 2124-2134.).

The preliminary data on 12 chemicals are promising (no misclassification), but further evaluation using more chemicals is required. In its current format, the test is expensive and rather complicated which may hamper its application by industry. More work is required to refine the test to make it more attractive for industrial use.

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